HYALURONAN AS A CYTOTOXIC AGENT, DRUG PRE-SENSITIZER AND CHEMO-SENSITIZER IN THE TREATMENT OF DISEASE

FIELD OF THE INVENTION

5   The present invention relates to the enhancement of bioavailability of chemotherapeutic agents for the treatment of disease.   In particular the present invention relates to the use of hyaluronan either alone or in combination with a chemotherapeutic agent to enhancement

10   the bioavailability of the chemotherapeutic agent for treatment of disease.   The present invention also relates to the treatment of a drug resistant disease whereby the drug resistance is overcome or alleviated with the use of hyaluronan either alone or in combination with a

15   chemotherapeutic agent.

BACKGROUND TO THE INVENTION

       Many diseases that afflict animals, including humans, are treated with chemotherapeutic agents.   For

20   example, chemotherapeutic agents have proven valuable in the treatment of neoplastic disorders including connective or autoimmune diseases, metabolic disorders, and dermatological diseases, and many of these agents are highly effective and do not suffer from any bioavailability

25.   problems.

       Proper use of chemotherapeutic agents requires a thorough familiarity with the natural history and pathophysiology of the disease before selecting the chemotherapeutic agent, determining a dose, and undertaking

30   therapy.   Each subject must be carefully evaluated, with attention directed toward factors which may potentiate toxicity, such as overt or occult infections, bleeding dyscrasias, poor nutritional status, and severe metabolic disturbances.   In addition, the functional condition of

35   certain major organs, such as liver, kidneys, and bone marrow, is extremely important.   Therefore, the selection of the appropriate chemotherapeutic agent and devising an

effective therapeutic regimen is influenced by the presentation of the subject. Such considerations affect the dosage and type of drug administered.

Unfortunately, not all chemotherapeutics are
5   readily useable. For example, some chemotherapeutic agents are inherently refractory in that animal cells do not readily respond to these agents, while other chemotherapeutics suffer from acquired resistance. For instance, it is well recognised that some subjects on
10   prolonged chemotherapy are forced to change chemotherapeutics as these become less efficacious with time. Moreover, some chemotherapeutics, while not affected by inherent or acquired resistance *per se*, are not effective in the treatment of certain diseases as they have
15   innate problems with bioavailability. One disease that is frequently affected by both cellular resistance and bioavailability problems is cancer.

Cancer is responsible for one in four deaths in Western society. While the rates of new cases of cancer
20   and deaths with cancer decreased in the United States and Canada between 1990-1994, the data show that 2,604,650 people in the United States died from cancer between 1990-1994, with more men (53%) than women (47%) affected. The most common cancer deaths were due to cancer of the lung
25   (728,641), colon and rectum (285,724), breast (218,786), and prostate (169,943).

Among women, the most common cancers are breast (31%), lung (12%), colon and rectum (12%), uterus (6%), and ovary (4%), with breast and ovarian cancer representing
30   approximately 35% of all cancers found in women. The majority of women diagnosed with these forms of cancer receive a combination of surgical, radiation therapy or chemotherapy.

Chemotherapeutic agents used to treat cancer can
35   be subdivided into several broad categories, including, (1) alkylating agents, such as mechlorethamine, cyclophosphamide, melphalan, uracil mustard, chlorambucil,

busulfan, carmustine, lomustine, semustine, streptozoticin, and decrabazine; (2) antimetabolites, such as methotrexate, fluorouracil, fluorodeoxyuridine, cytarabine, azarabine, idoxuridine, mercaptopurine, azathioprine, thioguanine, and

5   adenine arabinoside; (3) natural product derivatives, such as vinblastine, vincristine, dactinomycin, daunorubicin, doxorubicin, mithramycin, taxanes (e. g., paclitaxel) bleomycin, etoposide, teniposide, and mitomycin C ; and (4) miscellaneous agents, such as hydroxyurea, procarbezine,

10 mititane, and cisplatinum.

Important cancer chemotherapeutic agents (with the usual effective dosage) to which clinical multidrug-resistance has been observed include vinblastine (0.1 mg per kilogram per week), vincristine (0.01 mg per kilogram

15 per week), etoposide (35 to 50 mg per square meter per day), dactinomycin (0.15 mg per kilogram per day), doxorubicin (500 to 600 mg per square meter per week), daunorubicin (65 to 75 mg per square meter per week), and mithramycin (0.025 mg per kilogram per day).

20   It is well appreciated by those skilled in the field that, at present, there are no effective means of overcoming cellular resistance to chemotherapeutic agents. More importantly there are no practical means of increasing bioavailability of chemotherapeutics without concomitant

25 increase in toxicity or side effects. Accordingly, there is a requirement for means of overcoming or at least alleviating the problems associated with acquired or inherent cellular resistance as well as means of increasing bioavailability of chemotherapeutics.

30   The applicant has previously investigated the usefulness of hyaluronan (HA) as a drug delivery vehicle for chemotherapeutics, and found that HA was useful when co-administered with these drugs. International patent application no. PCT/AU00/00004 was filed covering this

35 invention, and is incorporated in its entirety herein by reference. HA, also known as hyaluronic acid, is a naturally occurring polysaccharide comprising linear-chain

polymers, which is found ubiquitously throughout the animal kingdom. HA is highly water-soluble, making it an ideal drug delivery vehicle for biological systems.

Subsequent to the filing of International patent
5   application no. PCT/AU00/00004, the applicant surprising found that HA could act as a sole agent. It was found that HA could exert a cytotoxic effect on human breast cancer cells, as well as pre-sensitizing cells so that they became more susceptible to chemotherapeutic agents. The present
10   invention therefore provides methods whereby cells that were, or had become resistant to chemotherapeutic agents could be effectively treated. More importantly, by using the disclosed methods it is possible to decrease the dosages of chemotherapeutic agents without decreasing the
15   efficacy to the subject. The methods of the invention include administering hyaluronan either alone in conjunction with a chemotherapeutic agent.

The present invention is based upon the discovery that hyaluronan, derivatives, analogues, and salts thereof,
20   not only inhibit cells *per se*, but also allows the safe administration of selected chemotherapeutic agents at standard or lower doses thought to be less effective, to treat subjects including human subjects. *In vivo* administration of hyaluronan in combination with
25   chemotherapeutic agents also enhances the therapeutic effect of these agents against cells that are refractory, thus preventing the subsequent emergence of multidrug resistance.

Diseased cells such as cancer cells often have
30   more permeable membranes due to an alteration in the membrane potential, or increased receptor status which can alter the regulation of their intracellular molecule transport which can result in cell swelling (Lang *et al*, 1993). While the applicant does not wish to be bound by
35   any theory they postulate that there are several mechanisms that could explain the cellular effect that HA is exerting both as a sole agent, and as a pre-treatment for

therapeutic agents:

1). When HA is bound to CD44, RHAMM and the scavenger receptor bound, the nett negative charge of HA alters the membrane potential of the cell resulting in an increase in cell permeability consequently enabling a greater flux of drug into the diseases cell.

2). When HA is bound to diseased cells such as tumour cells and internalised there could be a hyperosmotic effect resulting in cell lysis.

3). HA could exert oxidative membrane damage resulting in apoptosis.

4). HA internalisation could elevate the mitochondrial membrane potential which could result in cell death or increased drug retention.

Since HA is administered at satuarable levels, there would be a constant internalisation of the glycosaminoglycan which means that any therapeutic agent which is in an equilibrium within the volumetric domain of the HA is co-internalised resulting in a concentrated intracellular release of the drug

SUMMARY OF INVENTION

In its broadest aspect the present invention provides a method of treating a subject in need thereof comprising the step of administering to said subject a therapeutically effective amount of hyaluronan in conjunction with a chemotherapeutic agent such that said chemotherapeutic agent is more effective than when administered alone.

The present invention also provides a method of enhancing the bioavailability of a chemotherapeutic agent comprising the step of administering to a subject in need thereof a therapeutically effective amount of hyaluronan.

Hyaluronan can be used to significantly enhance the bioavailability of any administered chemotherapeutic agent. Preferably, the chemotherapeutic agent that is administered is selected from the group consisting of

carmustine (BCNU), chlorambucil (Leukeran), cisplatin
(Platinol), Cytarabine, doxorubicin (Adriamycin),
fluorouracil (5-FU), methoxetrate (Mexate), CPT111,
etoposide, plicamycin (Mithracin) and taxanes such as, for
5   example, paclitaxel.

       In yet another embodiment, the invention provides
a method of treating or preventing multidrug resistance or
drug-resistant cells comprising the step of administering a
therapeutically effective amount of hyaluronan, prior to,
10   together with, or subsequent to the administration of a
chemotherapeutic agent.

       As described more fully below, administration of
hyaluronan and a chemotherapeutic agent results in the
suppression of tumor growth by at least 50%; preferably
15   60%; and, more preferably, greater than 70%. Accordingly,
the elimination of tumor growth and proliferation
eliminates the production of multidrug resistant cells
reducing the recurrence of cancer and increasing the
efficacy of chemotherapeutic treatments.

20   The present invention further provides a
pharmaceutical composition for increasing the sensitivity
of cells to chemotherapeutic agents comprising hyaluronan.
The hyaluronan and/or chemotherapeutic agent may also be
administered together with a further pharmaceutical
25   carrier.

       The present invention also provides a method of
treating cancer cells comprising the step of administering
to a patient in thereof a therapeutically effective amount
of hyaluronan.

30   Typically said cancer cells are resistant to
chemotherapeutic drugs.

       In a further aspect of the present invention
there is provided a method of overcoming cellular
resistance, comprising the step of administering a
35   therapeutically effective amount of HA.

       Throughout the description and claims of this
specification, the word "comprise" and variations of the

word, such as "comprising" and "comprises", means "including but not limited to" and is not intended to exclude other additives, components, integers or steps.

5   BRIEF DESCRIPTION OF THE FIGURES

Figures 1 shows exponentially growing breast cancer cells exposed to 750,000 dalton HA for 24h at which stage the cells were photographed. At 10ng/ml there was a reduction in cell number, but no difference in morphology

10 was noted. At 100ng/ml and 1µg/ml the cells appeared top be undergoing a osmotic response where the cells appeared to swell. At 2mg/ml and 5mg/ml the cells became granular and the plasma membrane was "pitted" possibly indicating an osmotic response and/or the commencement of cell death.

15 Figure 2a-2f shows exponentially growing breast cancer cells that were exposed to 750,000 dalton HA for 30 min, 1h, or 24h at which stage the cells were varying concentrations of adriamycin. These figures also illustrate the effect of HA/drug co-incubation for the

20 period of 1 or 3 days. These diagrams illustrate that HA can "pre-sensitise" and/or chemosensitise cells to therapeutic drugs.

Figures 3a-3d shows exponentially growing breast cancer cells exposed to varying concentrations of 750,000

25 dalton hyaluronan for 1h, 24h or 3 days followed by treatment with 40nM Adriamycin for varying time periods of 1h, 24h or 3 days. These figures show that a wide concentration range of hyaluronan can act as a chemosenitiser or exert a cytotoxic effect.

30 Figure 4 shows that there was no treatment toxicity noted throughout the 6-week study. In comparison to the 5-FU treatment group the mice receiving HA therapy, that is as a sole agent or as a chemosensitizer, demonstrated enhanced well being where the animal did not

35 loose weight, but maintained its body mass.

Figure 5 shows that at the end of the 6 week study, tumour mass was determined where the HA

chemosensitizing therapy had significantly smaller tumours than the saline group, HA and 5-FU groups (p=0.005). HA as a sole agent also demonstrated its effect by reducing the primary tumour mass in comparison to the saline control.

5   No significant differences in tumour response were noted in the initial 2 weeks of treatment, but thereafter the HA followed by 5-FU tumour growth was retarded in comparison to the other treatment groups. During the 6 weeks of treatment interesting differences were noted in the number

10 of tumour doubling cycles. Mice receiving the saline treatment underwent an average of 4 tumour doublings, while the incorporation of HA into the treatment regimen significantly increased the tumour doubling time where HA/5-FU animals underwent an average of one tumour doubling

15 cycle, once again highlighting the effect of HA on 5-FU cytotoxicity.

Figure 6 shows that the co-administration of HA resulted in a significant reduction in non-lymphoid metastasis. With the exception of the mice receiving the HA

20 therapy, new tumours were observed around the neck or underarm region of the area adjacent to the primary tumour.

DETAILED DESCRIPTION OF THE INVENTION

The methods and compositions of the invention are

25 useful for increasing the sensitivity of cells to chemotherapeutic agents such as, for example, anti-cancer agents like paclitaxel, analgesics, opiates, hormones or antibiotics and the like. In particular the methods and compositions of the invention are useful for increasing the

30 sensitivity of cells associated with cellular proliferative disorders (eg., a neoplasm). By increasing the efficacy without concomitant toxicity to non-cancer cells the invention provides methods and compositions useful for treating tumors and preventing or reducing the chances of

35 relapse and death as a result of cytotoxicity. In addition, the invention eliminates or reduces the number of multidrug resistant cells by eliminating cancer cells prior

to any mutation inducing a multidrug resistant phenotype. Accordingly, by reducing multi-drug resistant tumor cells from arising, the invention satisfies the shortcomings of current therapeutic modalities.

5   The term "subject" as used herein refers to any animal having a disease or condition which requires treatment with a chemotherapeutic agent wherein the chemotherapeutic agent has reduced efficacy relative to that desired. Preferably the subject is suffering from a

10   cellular proliferative disorder (eg., a neoplastic disorder). Subjects for the purposes of the invention include, but are not limited to, mammals (eg., bovine, canine, equine, feline, porcine) and preferably humans.

By "cell proliferative disorder" is meant that a

15   cell or cells demonstrate abnormal growth, typically aberrant growth, leading to a neoplasm, tumor or a cancer.

Cell proliferative disorders include, for example, cancers of the breast, lung, prostate, kidney, skin, neural, ovary, uterus, liver, pancreas, epithelial,

20   gastric, intestinal, exocrine, endocrine, lymphatic, haematopoietic system or head and neck tissue.

Generally, neoplastic diseases are conditions in which abnormal proliferation of cells results in a mass of tissue called a neoplasm or tumor. Neoplasms have varying

25   degrees of abnormalities in structure and behaviour. Some neoplasms are benign while others are malignant or cancerous. An effective treatment of neoplastic disease would be considered a valuable contribution to the search for cancer preventive or curative procedures.

30   The methods of this invention involve in one embodiment, (1) the administration of hyaluronan, prior to, together with, or subsequent to the administration of a chemotherapeutic agent; or (2) the administration of a combination of hyaluronan and a chemotherapeutic agent.

35   As used herein, the term "therapeutically effective amount" is meant an amount of a compound of the present invention effective to yield a desired therapeutic

response. For example to prevent cancer or treat the symptoms of cancer in a host or an amount effective to treat cancer.

The specific "therapeutically effective amount"
5   will, obviously, vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of
10   the compounds or its derivatives.

As used herein, a "pharmaceutical carrier" is a pharmaceutically acceptable solvent, suspending agent or vehicle for delivering the hyaluronan and/or chemotherapeutic agent to the animal or human. The carrier
15   may be liquid or solid and is selected with the planned manner of administration in mind.

As used herein, "cancer" refers to all types of cancers or neoplasm or malignant tumours found in mammals. Cancer includes sarcomas, lymphomas and other cancers. The
20   following types are examples, but are, but is not intended to be limited to these particular types of cancers: prostate, colon, breast, both the MX-1 and the MCF lines, pancreatic, neuroblastoma, rhabdomysarcoma, home, lung, murine, melanoma, leukemia, pancreatic, melanoma, ovarian,
25   brain, head & neck, kidney, mesothelioma, sarcoma, Kaposi's, sarcoma, stomach, and uterine.

As used herein, the term "cell" include but is not limited to mammalian cells (eg., mouse cells rat cells or human cells).
30   The hyaluronan and/or chemotherapeutic agents may be administered orally, topically, or parenterally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes
35   subcutaneous injections, aerosol, intravenous, intramuscular, intrathecal, intracranial, intrasternal injection or infusion techniques.

The present invention also provides suitable topical, oral, and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compounds of the present invention may be
5   administered orally as tablets, aqueous or oily suspensions, lozenges, troches, powders, granules, emulsions, capsules, syrups or elixirs. The composition for oral use may contain one or more agents selected from the group of sweetening agents, flavouring agents,
10   colouring agents and preserving agents in order to produce pharmaceutically elegant and palatable preparations. The tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets.
15   These excipients may be, for example, (1) inert diluents, such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; (2) granulating and disintegrating agents, such as corn starch or alginic acid; (3) binding agents, such as starch, gelatin or acacia; and
20   (4) lubricating agents, such as magnesium stearate, stearic acid or talc. These tablets may be uncoated or coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay
25   material such as glyceryl monostearate or glyceryl distearate may be employed. Coating may also be performed using techniques described in the U. S. Pat. Nos. 4,256,108; 4,160,452; and 4,265,874 to form osmotic therapeutic tablets for control release.
30   The hyaluronan as well as the chemotherapeutic agents useful in the method of the invention can be administered, for in vivo application, parenterally by injection or by gradual perfusion over time independently or together. Administration may be intravenously,
35   intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally. For in vitro studies the agents may be added or dissolved in an appropriate

biologically acceptable buffer and added to a cell or
tissue.

Preparations for parenteral administration
include sterile aqueous or non-aqueous solutions,
suspensions, and emulsions. Examples of non-aqueous
solvents are propylene glycol, polyethylene glycol,
vegetable oils such as olive oil, and injectable organic
esters such as ethyl oleate. Aqueous carriers include
water, alcoholic/aqueous solutions, emulsions or
suspensions, including saline and buffered media.
Parenteral vehicles include sodium chloride solution,
Ringer's dextrose, dextrose and sodium chloride, lactated
Ringer's intravenous vehicles include fluid and nutrient
replenishers, electrolyte replenishers (such as those based
on Ringer's dextrose), and the like. Preservatives and
other additives may also be present such as, for example,
anti-microbials, anti-oxidants, chelating agents, growth
factors and inert gases and the like.

It is envisioned that the invention can be used
to treat pathologies associated cell proliferative
disorders, including, for example, neoplasms, cancers (eg.,
cancers of the breast, lung, prostate, kidney, skin,
neural, ovary, uterus, liver, pancreas, epithelial,
gastric, intestinal, exocrine, endocrine, lymphatic,
haematopoietic system or head and neck tissue), fibrotic
disorders and the like.

The methods and compounds of the invention may
also be used to treat other diseases associated with
chemotherapeutic treatment such as neurodegenerative
disorders, hormonal imbalance and the like. Therefore, the
present invention encompasses methods for ameliorating a
disorder associated with cell proliferation, neoplasms,
cancers and the like, including treating a subject having
the disorder, at the site of the disorder, with hyaluronan
and a chemotherapeutic agent in an amount sufficient to
inhibit or ameliorate the cell's proliferation or the
disorder. Generally, the terms "treating", "treatment" and

the like are used herein to mean affecting a subject,
tissue or cell to obtain a desired pharmacologic and/or
physiologic effect. The effect may be prophylactic in
terms of completely or partially preventing a cell
5   proliferative disorder or sign or symptom thereof, and/or
may be therapeutic in terms of a partial or complete cure
for a disorder and/or adverse effect attributable to, for
example, aberrant cell proliferation. "Treating" as used
herein covers any treatment of, or prevention of a cell
10 proliferative disorder in a vertebrate, a mammal,
particularly a human, and includes: (a) preventing the
disorder from occurring in a subject that may be
predisposed to the disorder, but has not yet been diagnosed
as having it; (b) inhibiting the disorder, i. e., arresting
15 its development; or (c) relieving or ameliorating the
disorder, i. e., cause regression of the disorder.

The invention includes various pharmaceutical
compositions useful for ameliorating cell proliferative
disorder, including neoplasms, cancers and the like. The
20 pharmaceutical compositions according to one embodiment of
the invention are prepared by bringing hyaluronan,
analogue, derivatives or salts thereof and one or more
chemotherapeutic agents or combinations of hyaluronan and
one or more chemotherapeutic agents into a form suitable
25 for administration to a subject using carriers, excipients
and additives or auxiliaries. Frequently used carriers or
auxiliaries include magnesium carbonate, titanium dioxide,
lactose, mannitol and other sugars, talc, milk protein,
gelatin, starch, vitamins, cellulose and its derivatives,
30 animal and vegetable oils, polyethylene glycols and
solvents, such as sterile water, alcohols, glycerol and
polyhydric alcohols. Intravenous vehicles include fluid
and nutrient replenishers. Preservatives include
antimicrobial, anti-oxidants, chelating agents and inert
35 gases. Other pharmaceutically acceptable carriers include
aqueous solutions, non-toxic excipients, including salts,
preservatives, buffers and the like, as described, for

instance, in Remington's Pharmaceutical Sciences, 15th ed.
Easton: Mack Publishing Co., 1405-1412,1461-1487 (1975) and
The National Formulary XIV., 14th ed. Washington: American
Pharmaceutical Association (1975), the contents of which

5   are hereby incorporated by reference. The pH and exact
concentration of the various components of the
pharmaceutical composition are adjusted according to
routine skills in the art. See Goodman and Gilman's The
Pharmacological Basis for Therapeutics (7th ed.).

10   The pharmaceutical compositions are preferably
prepared and administered in dose units. Solid dose units
are tablets, capsules and suppositories. For treatment of
a subject, depending on activity of the compound, manner of
administration, nature and severity of the disorder, age

15   and body weight of the subject, different daily doses can
be used. Under certain circumstances, however, higher or
lower daily doses may be appropriate. The administration
of the daily dose can be carried out both by single
administration in the form of an individual dose unit or

20   else several smaller dose units and also by multiple
administration of subdivided doses at specific intervals.
             The pharmaceutical compositions according to the
invention may be administered locally or systemically in a
therapeutically effective dose. Amounts effective for this

25   use will, of course, depend on the severity of the disease
and the weight and general state of the subject.
Typically, dosages used *in vitro* may provide useful
guidance in the amounts useful for *in situ* administration
of the pharmaceutical composition, and animal models may be

30   used to determine effective dosages for treatment of
particular disorders. Various considerations are described,
eg., in Langer, Science, 249: 1527, (1990). Formulations
for oral use may be in the form of hard gelatin capsules
wherein the active ingredient is mixed with an inert solid

35   diluent, for example, calcium carbonate, calcium phosphate
or kaolin. They may also be in the form of soft gelatin
capsules wherein the active ingredient is mixed with water

or an oil medium, such as peanut oil, liquid paraffin or
olive oil.

Aqueous suspensions normally contain the active
materials in admixture with excipients suitable for the
5   manufacture of aqueous suspension. Such excipients may be
(1) suspending agent such as sodium carboxymethyl
cellulose, methyl cellulose, hydroxypropylmethylcellulose,
sodium alginate, polyvinylpyrrolidone, gum tragacanth and
gum acacia; (2) dispersing or wetting agents which may be
10   (a) naturally occurring phosphatide such as lecithin; (b) a
condensation product of an alkylene oxide with a fatty
acid, for example, polyoxyethylene stearate; (c) a
condensation product of ethylene oxide with a long chain
aliphatic alcohol, for example, heptadecaethylenoxycetanol;
15   (d) a condensation product of ethylene oxide with a partial
ester derived from a fatty acid and hexitol such as
polyoxyethylene sorbitol monooleate, or (e) a condensation
product of ethylene oxide with a partial ester derived from
fatty acids and hexitol anhydrides, for example
20   polyoxyethylene sorbitan monooleate.

The pharmaceutical compositions may be in the
form of a sterile injectable aqueous or oleagenous
suspension. This suspension may be formulated according to
known methods using those suitable dispersing or wetting
25   agents and suspending agents which have been mentioned
above. The sterile injectable preparation may also a
sterile injectable solution or suspension in a non-toxic
parenterally-acceptable diluent or solvent, for example, as
a solution in 1,3-butanediol. Among the acceptable
30   vehicles and solvents that may be employed are water,
Ringer's solution, and isotonic sodium chloride solution.
In addition, sterile, fixed oils are conventionally
employed as a solvent or suspending medium. For this
purpose, any bland fixed oil may be employed including
35   synthetic mono-or diglycerides. In addition, fatty acids
such as oleic acid find use in the preparation of
injectables.

Hyaluronan together with a chemotherapeutic agent
of the present invention may also be administered in the
form of liposome delivery systems, such as small
unilamellar vesicles, large unilamellar vesicles, and
5   multilamellar vesicles. Liposomes can be formed from a
variety of phospholipids, such as cholesterol,
stearylamine, or phosphatidylcholines.

Dosage levels of the compounds of the present
invention are of the order of about 0.5mg to about 10mg per
10   kilogram body weight, with a preferred dosage range between
about 5mg to about 20mg per kilogram body weight per day
(from about 0.3gms to about 1.2gms per patient per day).
The amount of active ingredient that may be combined with
the carrier materials to produce a single dosage will vary
15   depending upon the host treated and the particular mode of
administration. For example, a formulation intended for
oral administration to humans may contain about 5mg to 1g
of an active compound with an appropriate and convenient
amount of carrier material which may vary from about 5 to
20   95 percent of the total composition. Dosage unit forms
will generally contain between from about 5mg to 500mg of
active ingredient.

It will be understood, however, that the specific
dose level for any particular patient will depend upon a
25   variety of factors including the activity of the specific
compound employed, the age, body weight, general health,
sex, diet, time of administration, route of administration,
rate of excretion, drug combination and the severity of the
particular disease undergoing therapy.
30   In addition, some of the compounds of the instant
invention may form solvates with water or common organic
solvents. Such solvates are encompassed within the scope of
the invention.

The compounds of the present invention may
35   additionally be combined with other compounds to provide an
operative combination. It is intended to include any
chemically compatible combination of chemotherapeutic

agents, as long as the combination does not eliminate the activity of the hyaluronan of this invention.

The invention will now be further described by way of reference only to the following non-limiting
5   examples. It should be understood, however, that the examples following are illustrative only, and should not be taken in any way as a restriction on the generality of the invention described above. In particular, while the invention is described in detail in relation to cancer, it
10   will be clearly understood that the findings herein are not limited to treatment of cancer. For example, HA may be used for treatment of other conditions.

Example 1   Preparation of Hyaluronan and 5-Fluorouracil
15   Solutions
HA used in all of the *in vitro* and *in vivo* studies were obtained from Kyowa Hakko Kogyo (Yamaguchi, Japan). 5-FU was obtained from Sigma, St. Louis, USA. And Adraimycin from Cytomix, Northcote, Melbourne, Australia.
20   A standard profile of the HA used is shown in Table 1.

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Table 1

Specification Sheet For Hyaluronan Bulk Dried Powder

TEST	SPECIFICATION
1. Description	White or cream coloured powder or granules, odourless
2. Identification (IR Spectrum)	Conforms to Reference Standard
3. pH (1% solution)	5.0 to 7.0
4. Loss on Drying	NMT 10.0%
5. Residue on Ignition	15.0 to 19.0%
6. Protein Content	NMT 0.1%
7. Heavy Metals	NMT 20ppm
8. Arsenic	NMT 2ppm
9. Sodium Hyaluronate Assay	97.0-102.0% (dried basis)
10. Intrinsic Viscosity	10.0-14.5dL/g
11. Total Aerobic Microbial Count (USP 23)	NMT 50 CFU/gram
12. *Staphylococcus aureus* (USP 23)	Absent
13. *Pseudomonas aeruginosa* (USP 23)	Absent
14. Yeasts and Moulds (USP 23)	NMT 50 CFU/gram
15. Bacterial Endotoxin (LAL)(USP23)	NMT 0.07 EU/mg

5

A 10mg/ml stock of HA solution was prepared by dissolving
desiccated HA (modal M_r 7.5x10^{5}kDa,) in pyrogen-free
injection grade water. To ensure a homogenous solution the
HA was dissolved overnight at 4°C followed by thorough
5   vortexing. To ensure that the HA had maintained its
molecular weight during the preparation of the stock
solution, the solution was analysed on a Sephacryl S-1000
size exclusion gel with column specifications of 1.6cm x
70cm, sample size 2ml, flow rate 18ml/h and 2ml fraction
10   size. Hyaluronan was detected in column fractions by the
uronic acid assay.

The uronic acid assay was used to detect the
presence of hyaluronan qualitatively from the fractions
collected from the gel filtration chromatography procedure.
15   A 25µl aliquot of each fraction was then transferred into a
96 well plate. 250µl of a carbazole reagent (3M
carbazole/0.025M borate in H$_2$SO$_4$) was then added to these
fractions. The 96 well plate was incubated for 45-60min at
80°C. A Dynatech MR7000 plate reader with a 550nm filter
20   was used to read the 96 well plate. The absorbance was
considered to be significant when it was >3 standard
deviations above the background absorbance. The background
was calculated by taking an equal number of sample points
before and after V_o and V_t where the average number taken
25   was 16 (Fraser et al. 1998).

A stock solution of 5-FU was prepared by
dissolving powdered 5-FU in 0.1M NaOH (pH 8.9) and brought
to a concentration of 1mg/ml with pyrogen-free injection
grade 0.9%w/v NaCl. The stock solution was filtered
30   through a 0.22µm filter to ensure sterility. The 5-FU was
diluted by adding the required volume of stock solution to
the cell-line specific growth medium as specified above.

A 10mg/ml solution of adriamycin in 0.9% NaCl was
obtained from Cytomix.

35

Example 2   Testing the Effect of Hyaluronan on Cancer
                 Cell Morphology

Human breast adenocarcinoma cell lines MDA-MB-
468, MDA-MB-435 and MDA-MB-231 were selected based on HA
binding affinity (Culty *et al*, 1994), and the expression of
the HA receptors of CD44 and RHAMM (Wang *et al*, 1996). The
characteristics of these cell lines are shown in Table 2.

Table 2

Hyaluronan Binding And Receptor Expression Of Human Mammary Carcinoma Cell Lines

Cell Line	Type of breast cancer	Degree of HA Binding[a]	HA Receptor Expression[b]	
			CD44	RHAMM
MDA-MB-231	adenocarcinoma	++	+++	+++
MDA-MB-468	adenocarcinoma	++++	++++	++
MDA-MB-435	ductal carcinoma	+	+++	ND

a: Culty *et al*, 1994

b: Wang *et al*, 1996

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Cell lines MDA-MB-468, MDA-MB-435 and MDA-MB-231 were routinely grown and subcultured as a monolayer in 175cm^2 culture flasks in Leibovitz L-15 Medium supplemented with 10% Foetal calf serum (FCS) and antibiotic/antimycotic

5   reagents at 37°C in humidity controlled incubator with 100% (v/v) air.

Leibovitz-L-15 with glutamine (10x concentrate), RPMI (10x concentrate), Eagles basal medium (EBM, 10x concentrate), 20mM HEPES, 0.09% w/v bicarbonate, Hanks'

10   Balanced Salt Solution (HBSS, 10x concentrate) and Dulbecco's Phosphate Buffered Saline without calcium and magnesium (PBS, 10x concentrate) were purchased from Sigma (St Louis, Mo., USA). Powder concentrates were dissolved in the required volume of reverse osmosis deionised

15   pyrogen-free distilled water to make a single strength solution, sterilised by 0.22 μm high pressure filtration (Millipore Corporation, MA. U.S.A.), and stored at 4°C. FCS) were purchased from the CSL Ltd., Australia. FCS was stored at -20°C. Antibiotic/antimycotic solution (100X

20   concentrate) containing 10,000 units penicillin, 10mg streptomycin and 25μg amphotericin U/ml was obtained from Sigma (St Louis, USA). Trypsin/EDTA solution (10X concentrate) containing 5g porcine trypsin and 2g EDTA/L in 0.9%w/v sodium chloride was obtained from Sigma (St Louis,

25   Mo., USA). All breast cancer cell lines were purchased from American tissue culture collection (Rockville, USA). All plastic disposable culture vessels were purchased from Greiner (Austria). Eight-welled, tissue culture microscope slides were obtained from Linbro (Flow Laboratories, VA,

30   USA).

For the tests, MDA MB-468, MDA MB-231 and MDA MB-435 cell line were grown in 90% Leibovitz L-15 medium supplemented with 10% FCS. When confluent the cultures were washed 1 X in HBSS and trypsinised in 0.25%

35   trypsin/0.05% EDTA. The cell suspensions were counted with an automated cell counter (ZM-2 Coulter Counter) by adding 15mL saline + 0.2ml of cell suspension.

Cells were resuspended to a number of:
MDA MB-468: 25,000 cell/ml of media
MDA MB-231: 12,000 cell/ml of media
MDA MB-435: 12,000 cell/ml of media

5   The cells were plated into 48-well plates (1cm^2 surface
area) by adding 1ml of cell suspension per well.
Cells were allowed to attach for 24h, before the media was
removéd, monolayers washed. The test media was; growth
media containing 0-1μM adriamycin or 5-fluorouracil with or

10 without the addition of 0-1μM of HA (modal Mw 750,000).
The cells were exposed to the several combinations of HA
and drugs for different times and at different
concentrations (Table 3).

Table 3

Incubation Conditions for Hyaluronan and Drugs with Human
Breast Cancer Cells

Sequence of HA/Drug Addition	HA Incubation Time	Drug Incubation Time	Growth Time
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	30min	1h	1 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	1h	1h	1 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	24h	1h	1 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	24h	24	1 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	30min	1h	3 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	1h	1h	3 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	24h	1h	3 day
0-1μM HA, media wash, 0-1μM drug, media wash, grow drug-free	24h	24	3 day
0-1μM drug/100nM HA		30min	1 day
0-1μM drug/100nM HA		1h	1 day
0-1μM drug/100nM HA		24	1 day
0-1μM drug/100nM HA		30min	3 days
0-1μM drug/100nM HA		1h	3 days
0-1μM drug/100nM HA		24	3 days
0-1μM HA	30min		1 day
0-1μM HA	1h		1 day
0-1μM HA	24		1 day
0-1μM HA	30min		3 days
0-1μM HA	1h		3 days
0-1μM HA	24		3 days
0-1μM HA	3 days		3 days

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After the incubation and growth periods the cell monolayers
were washed with HBSS and trypsinised in 0.25%
trypsin/0.05% EDTA. The cell suspensions were counted with
an automated cell counter (ZM-2 Coulter Counter) by adding
5 15mL saline + 0.2ml of cell suspension. Results were
expressed as % of no drug control which was calculated as:

$$\frac{\text{Cell count X 100}}{\text{Cells in no drug control}}$$

10

Or depending on the experiment as % of drug control,
calculated as:

$$\frac{\text{Cell count X 100}}{\text{Cells in drug control}}$$
15

        Exponentially growing human breast cancer cells
MDA MB 231 as described in example 2 were incubated with 0-
5mg/ml HA (modal Mr 750,000D) for 24h. At 24 h the cells
20 were counted and photographed with CPR, 1600 film rolls
from Eastman Kodak Company, Rochester, USA.
        When HA was incubated with breast cancer cells
for 30min, 1h, 24h or 3 days a varied response was
observed, where the reduction in breast cancer cell number
25 ranged from 0-29% (See Table 4).

Table 4

Cytotoxic Effect of HA on Human Breast Cancer Cell Lines

Exposure Time	Cell Line MDA-MB 468	Cell Line MDA-MB 231	Cell Line MDA-MB 435
3 days 100nM	-29%	-23%	-22%
1h 100nM	+3%	-21%	-4%
30min 100nM	-5%	-27%	-12%
30min 500nM	-22%		0
30min 1000nM	+2%	-26%	ND
24h 100nM	-5%	-8%	-12%

*Figures are the mean of 2-3 separate determinations

When human breast cancer cells were incubated with HA specific morphological changes (See Figure 1) were also observed such as swelling of the plasma membrane, greater granularity of cytosolic components.

5   When human breast cancer cells were exposed to HA for 30min, 1h, 24h or 3 days followed by exposure toadriamycin, it became evident that HA ehanced the cytotoxicity of the drug (Figure 3 & Table 5).

Table 5

Effect of HA on Adriamycin Cytotoxicity in Breast Cancer
Cell Lines

Treatment	IC_{50} MDA-MB 468	IC_{50} MDA-MB 231	IC_{50} MDA-MB 435
3 day drug exposure	3 to 12	4 to 5	10
1h drug/HA, 3 days drug-free	40	2 to 8	0
1h drug, 3 days drug-free	20 to 40	3 to 9	6 to 10
30min 100nm HA, 1hr drug, 3 days drug-free	2 to 20	2 to 6	4 to 40
30min 100nM HA, 3 day drug exposure	3 to 18	2 to 4	2 to 8
30min 500nM HA, 3 day drug exposure	3 to 9	2 to 8	2 to 4
30min 1000nM HA, 3 day drug exposure	1 to 10	2 to 8	1 to 5
24h 100nM HA, 3 day drug exposure	8 to 12	13	24
24h 100nM HA, 1h drug exposure, drug-free 3 days	50 to 60	9	21

All figures represent the range of 2-3 separate experiments, where the
numerical values are the multiplication factor decrease in IC_{50} which
is exerted by the addition of HA to drug or pre-sensitization of
cancer cells with HA before the addition of drug.

<u>Example 3</u>   <u>Efficacy of Hyaluronan *In Vivo*</u>

      Based on the results from the *in vitro* drug
sensitivity experiments in Example 2, evaluation of the
treatment efficacy of hyaluronan as a sole agent, and as a
5   chemosensitizer in the treatment human breast carcinomas *in
vivo* was undertaken.

      From the results in Example 2 the carcinoma cell
line MDA-MB-468 was selected as the cancer cell inoculant
for the generation of any nude mouse human tumour
10 xenografts. Cells were routinely grown and subcultured as
a previously described in Example 2. For injection into
mice, cells were grown to 100% confluency, trypsinised in
0.025% trypsin/0.01% EDTA solution, washed twice by
centrifugation in a Beckman TJ-6 bench centrifuge at $400g_{av}$
15 for 10min, counted using a Model-ZM Coulter counter and
resuspended in serum-free Leibovitz L-15 medium at 1×10^8
cells/ml.

      Six to eight weeks old athymic CBA/WEHI nude
female mice, purchased from the Walter and Eliza Hall
20 Research Institute, Melbourne Australia, were maintained
under specific pathogen-free conditions, with sterilised
food and water available *ad libitum*. Each mouse received
one injection containing 5×10^6 cells in 50µl. The cells
were injected with a 26 gauge needle into the mammary fat
25 pad directly under the first nipple (Lamszus *et al*, 1997).
Tumour measurements were made weekly by measuring three
perpendicular diameters $(d_1 d_2 d_3)$. Tumour volume was
estimated using the formula:

30

$$(1/6)\pi \ (d_1 d_2 d_3)$$

Treatment with 5-FU $\pm$ HA was commenced approximately 4-8
weeks after the cancer cell inoculation. The mean tumour
size for mice used in each study is summarised in Table 6.

Table 6

Summary of Human Breast Cancer Tumours at
Commencement of Each Study

Study	Tumour volume (mean ± SEM)	Tumour as % of net body mass (mean ± SEM)
Efficacy: 6-week	$0.37 \pm 0.20 mm^3$	$0.19 \pm 0.10 mm^3$

Approximately 8 weeks after tumour induction two tumour-bearing mice were given a lethal dose of Nembutal. Within 3min of killing the mice, tumours were surgically removed and immediately fixed in 10% buffered formalin for

5   12h. The fixed tumour was dehydrated overnight in a series of 70-100% ethanol, followed by paraffin embedding from which 2-4μm sections were cut. The sections were placed on slides, de-waxed, and brought to water. Slides were washed 3 x 5min in PBS. Heterophile proteins were blocked by

10   incubation with 10% foetal calf serum for 10min, followed by a PBS rinse.

Secondary antibodies used in the visualisation of HA and HA synthase antibodies were purchased from Dako (California, U.S.A.). 3,3'-Diaminobenzidine (Sigma *Fast*

15   DAB) tablets were obtained from Sigma, St. Louis, USA.

The detection antibodies were applied for 60min at RT. The detection antisera or antibodies were against RHAMM , CD44H and CAE. The slides were washed 3x5min in PBS and endogenous peroxidase activity blocked by immersion in

20   0.3%H_2O_2 in methanol for 20min. Following a further PBS wash, the peroxidase-conjugated swine anti-rabbit secondary antiserum was applied for 60min at RT, followed by 3x5min washes in PBS. Sigma Fast 3,3'-Diaminobenzidine tablets (DAB) were prepared according to the manufacturer's

25   instructions and the DAB solution was applied for 5-10min at RT. The slides were washed in tap water for 10min, counterstained with haematoxylin, dehydrated and mounted.

Individual injections of 5-FU were prepared according to individual mouse masses, with the aim of

30   delivering 30mg/kg 5-FU in 50μl (equivalent to human therapeutic dose of 10.5mg/kg for a mean body weight of 60kg; Inaba *et al*, 1988). HA injection comprising a final HA concentration equivalent to 12.5mg/kg of mouse mass were prepared so that deliver of 12.5mg/kg HA in 50μl could be

35   effected. With this quantity of HA injected into the body, saturation kinetics would be observed for the period of the experimentation (Fraser *et al*, 1983).

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One of the most commonly used treatment regimens
for human breast cancer is cyclophosphamide, methotrexate
and 5-fluorouacil, which is administered on day 1 and 8 of
a 28 day cycle. In human breast cancer the initial
5   treatment regimen is for 6 cycles at which time the patient
condition is re-assessed, therefore we tried to simulate
the human treatment regimen as closely as possible by
exposing the mice to 6 cycles (6 months) of treatment in a
long term efficacy study and a 6 cycles (6 week) short term
10   efficacy study. Considering the life cycle of a mouse is
approximately 2 years we commenced both short-term and
long-term treatment protocols (see Table 7).

Table 7

Treatment Administration Protocols.

Treatment Group	Dosage	6-Week Study Treatment Regimen Bolus injection on Days
1. Saline	0.1ml of 0.9% saline (injection grade)	1 & 2 of 7 day cycle
2. HA	0.1ml containing: 12.5mg/kg HA	1 & 2 of 7 day cycle
3. 5-FU	0.1ml containing: 30mg/kg 5-FU	1 & 2 of 7 day cycle
4. HA followed by 5-FU	0.1ml containing: 12.5mg/kg HA or 30mg/kg 5-FU	1: HA 2: 5-FU 3: HA 4: 5-FU of 7 day cycle
5. HA	0.1ml containing: 12.5mg/kg HA	1: HA 3: HA of 7 day cycle
6. 5-FU	0.1ml containing: 30mg/kg 5-FU	2: HA 4: HA of 7 day cycle

5

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Mice were randomly divided into 7 groups of 8 animals per group for the short term study and 5 groups of 8 animals for the long term study (refer to Table 7 for dosage and treatment administration schedule).

5   The treatment was not extended over the 6 month regimen since it has been demonstrated that chemotherapy lasting more than six months has not generally been associated with greater benefit (Harris *et al*, 1992).

        Animals were weighed and tumour volumes measured

10 on the day of treatment application for long term study. In the 6-week study animals were weighed and tumour volumes measured on a daily basis. Animals were individually placed in an injection box, and the injections were administered via the tail vein. It has been experimentally proven that

15 stress can be a major factor in a patients response to chemotherapy (Shackney et al, 1978), therefore we ensured that equal numbers of mice were allocated to each cage, the animal number per cage varied from 5-8 depending on the stage of experimentation.

20   The experimental end-point occurred when the animal had to be euthanised due to degree of disease progression or when the 6 month (long term) or 6 week (short term) treatment regimen was completed. Due to the animal ethics guidelines the animals were monitored

25 fortnightly by an independent animal ethics officer who assessed the degree of disease progression. The following criteria were used to determine if an animal had reached the stage of experimental end-point of necessary death:

1).   Tumour mass was so large the animal was

30 immobilised;

2).   Animal was not eating or drinking and had experienced dramatic weight loss; or

3).   Tumour size was greater than 10% of body mass.

        At the experimental end-point the animals were

35 anaesthetized by a 0.1ml intra-peritoneal injection of Nembutal (60mg/ml), blood was collected followed by killing of the animals using cervical dislocation.

Immediately after killing the mouse the tumour, liver, heart, spleen, bladder, left and right kidneys, uterus, lungs, stomach, intestines, brain and lymph nodes were excised and placed in 4% formalin buffered with 0.06M
5   phosphate pH 7.5, and cetylpyridinium chloride, 1.0% w/v. The tissue was fixed for 16-24h before histological processing. Fixed tissue was dehydrated stepwise to 100% ethanol and embedded in paraffin blocks from which 2-4μm sections were placed on glass microscope slides. Staining
10   the tissue sections with a haematoxylin nuclear stain and eosin cytoplasmic stain highlighted any pathological features that could indicate treatment toxicity.

Nine to 11 lymph nodes were collected per animal, ensuring that all nodes which drained the tumour area were
15   collected. There are currently two methods used for the detection of lymph node metastasis

i)   routine haematoxylin and eosin staining of gross organ structure; and

ii)   immunohistochemistry using a cancer marker
20   such as carcinoembryonic antigen.

Both methods of metastasis detection were employed in this study. Not all commercially available CEA antibodies react with human breast cancer cells, so we tested the reactivity of 5 different antibodies (DAKO,
25   Amersham and KPL).

The haematoxylin and eosin stained lymph nodes were examined by Dr P. Allen (certified pathologist) where each node was microscopically examined for the presence of tumour cells. The CEA immunostained lymph nodes were
30   microscopically examined, where any positively stained nodes were counted and considered positive for lymph node metastasis.

Tumour volume was monitored on a daily or weekly basis by calliper measurements and tumour volume calculated
35   as previously described. At the end of the 6 week study, tumour mass was determined where the HA chemosensitizing therapy had significantly smaller tumours than the saline

group, HA and 5-FU groups (p=0.005) as seen in Fig 6. No
significant differences in tumour response were noted in
the initial 2 weeks of treatment, but thereafter the HA
followed by 5-FU tumour growth was retarded in comparison
5   to the other treatment groups. During the 6 weeks of
treatment interesting differences were noted in the number
of tumour doubling cycles. Mice receiving the saline
treatment underwent an average of 4 tumour doublings, while
the incorporation of HA into the treatment regimen
10   significantly increased the tumour doubling time where
HA/5-FU animals underwent an average of one tumour doubling
cycle, once again highlighting the effect of HA on 5-FU
cytotoxicity.

All animals displayed lymph node metastasis in
15   lymph nodes that were adjacent to the primary tumour. The
percentage of lymph node involvement (number of metastatic
nodes per animal) was greatly reduced by the HA followed by
5-FU, 5-FU and HA treatment, where the saline group
demonstrated a 6-fold increase in the amount of lymph node
20   involvement. The other treatment groups demonstrated a
significantly smaller percentage at 12.2 - 14.3 %
(Dunnett's Multiple Comparison Test, $p=<0.001$).

The co-administration of HA resulted in a
significant reduction in non-lymphoid metastasis. With the
25   exception of the mice receiving the HA therapy, new tumours
were observed around the neck or underarm region of the
area adjacent to the primary tumour.

Gastro-intestinal tract toxicity:
30   One of the most common toxic effects of 5-FU is
on the gastro-intestinal tract where haemorrhagic enteritis
and intestinal perforation can occur (Martindale, 1993).
Animals were monitored daily for GI tract upset such as
diarrhoea and weekly for more severe toxicity
35   manifestations such as weight loss. Weight loss was
monitored by calculating net body weight as estimated by
subtracting tumour weight, which was calculated as 1g x

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tumour volume (cm^3) as cited in Shibamoto *et al*, 1996. For demonstration of any weight changes the animal body weight was normalised to the body weight at the time of treatment commencement as

5

Body mass (ex tumour)- body mass at commencement of treatment (ex tumour)

Body mass at commencement of treatment (ex tumour)   X 100

No treatment toxicity was noted throughout the 6-week study. In comparison to the 5-FU treatment group the mice receiving HA therapy, that is as a sole agent or as a chemosensitizer, demonstrated enhanced well being where the animal did not loose weight, but maintained its body mass (Figure 4).

Blood Marrow suppression

As one of the major toxicities associated with 5-FU treatment is depression of the bone marrow and subsequent drop in white blood cells it was necessary to assess any treatment associated blood toxicity. Upon anaesthetising the animals, blood was collected from the heart or great vessels using a needle and syringe. Estimation of white blood cell number by making a 1/50 dilution of blood in mouse tenacity saline (M) and counting it on a haemocytometer. A differential blood count was performed by counting neutrophils, lymphocytes, and erythrocytes. The total estimation of blood cell sub-populations was compared to published data for mouse blood.

The total white cell count and sub-population differential were not significantly different, regardless of the treatment.

Effect of treatment on organ mass

To ensure that treatments did not induce organ atrophy or enlargement, the organs were removed and weighed during the post mortem. The mass of each organ was calculated as a % of the overall net body weight, and

compared to the organ masses of the saline only group (Group 1).

The overall patient survival time was calculated as the time (days or weeks) that the animal lived after the commencement of treatment. All animals in each treatment group completed the 6-week treatment program.

In relation to organ mass, the HA therapy did not result in any dramatic toxicity. Mice receiving 5-FU exhibited an enlarged spleen (61% increase in mass), while the co-administration of HA and 5-FU significantly counteracted this enlargement by 31% (student t-test, p<0.001). The 5-FU therapy resulted in a shrinkage of the uterus (22%), once again the HA/5-FU therapy reduced this toxic effect by 10% (student t-test, p=0.04). It was also clearly defined that the addition of HA to the treatment regimen, when co-administered or administered the day before, significantly decreased the primary tumour mass in comparison to the saline treatment group (student t-test, p=0.006). No other differences in organ mass were noted between treatments.

Example 4   Effect of Hyaluronan Concentration on the *In Vitro* Efficacy of 5-FU

MDA-MB 468, MDA-MB 435 and MDA-MB 231 cells were cultured as described in Example 2. When the cultures had reached 70-80% confluency they were washed in 1 X HBSS at 37°C and trypsinised in 10ml of 0.25% trypsin/0.05% EDTA until cells have fully detached. After add 1ml of FCS to neutralise trypsin the cells were counted, centrifuged at 1,200rpm for 5 min and resuspended as follows:

MDA-MB 231: 12,000cells/ml of media;
MDA-MB 468: 25,000cells/ml of media; and
MDA-MB 435: 12,000cells/ml of media.

Cells were then plated into 48-well plates and incubated in accordance with suppliers' instructions. After 24h media was removed and replaced with the following test media:

MDA-MB 468: 40nM adriamycin;

MDA-MB 231: 50nM adriamycin; and

MDA-MB 435: 10nM adriamycin

40nM Adriamycin media:450ml (Stock adriamycin is 1.7mM,
therefore 1,700,000/40 = 42,500; 450,000/42500=10.6ul of

5   1.7mM Adriamycin + 450 ml Media).

Stock HA was 700,000 daltons at 14.3µM HA

CONCLUSIONS

This study has definitively proven that HA, can

10 enhance the cytotoxicity of anti-cancer drugs, 5-FU and
Adriamycin, both *in vitro* and *in vivo*. More specifically:

1). As a sole agent HA can exert a cytotoxic
effect on cancer cells both in vitro and in vivo (Figure
5);

15   2). Evaluation of the therapeutic efficacy of HA
sole therapy or chemosensitizing therapy demonstrated that
it was not toxic to normal tissue and it did not enhance
the toxicity profile of the drug. In fact, mice receiving
the therapy displayed a significant weight gain over the 6-

20 week treatment period and a reduction in lymph node
metastasis. The co-administration of HA and 5-FU had a
dramatic effect on the reduction of the primary tumour
volume; and

3). Mice who had HA incorporated into the

25 treatment regimen did not display the formation of any
secondary tumour (Figure 6).

FUTURE STUDIES

Experiments are presently being conducted on the

30 use of HA for in vivo treatment of breast cancer. These
experiments are focusing on the effect of HA concentration
and molecular weight and on the cytotoxicity of adriamycin.
It is the aim of these studies to also establishing drug
and HA exposure time and regimens, as well as the mechanism

35 of action of HA, ie: receptor mediated transport and/or
effect on cell membrane. Further data on the role of HA in
chemosensitizing drug-resistant cancer cells will also be

collected.

Section 1:

            All studies will be conducted on breast cancer
5   cell lines that express differing levels of HA receptors,
    CD 44 and RHAMM. Cell lines to be tested, MDA-MB 435, MDA-
    MB 231, MDA-MB 468, ZRL-751 and several MDR-1 expressing
    breast cancer cell lines.
            Investigation of the effect of HA/adriamycin
10 exposure times and concentration on drug-resistant and
    drug-sensitive breast cancer cells. Four MDR-1 positive
    and 4 MDR-1 negative cell lines will be exposed to
    adriamycin at 1, 2.5, 5, 10, 20, 40, 60, 80 and 100nM, the
    following variables will be tested:
15 1).   1h drug ± 100nM HA exposure followed by 3 days of
    drug-free growth;
    2).   Constant drug exposure ± 100nM HA for 3 days
    30 min 100nM HA exposure, followed by drug for 1h, cells
    grown drug-free for 3 days;and
20 3).   24h 100nM HA exposure, followed by drug for 1h,
    cells grown drug-free for 3 days.
            These experiments will establish; optimal HA
    exposure times and regimens, magnitude of increased
    adriamycin cytotoxicity when combined with HA and whether
25 HA can overcome efflux pump resistance in breast cancer
    cells.
            To date the IC_{50} of adriamycin has been
    determined as 90nM. Using 90nM of adriamycin the HA
    (700kD) concentration will be varied to 1, 3, 10, 30, 100,
30 300nM, 1μM, 3μM, 10μM, 30μM and 100μM. The incubation
    variables to be tested are:
    1).   30min HA exposure followed by 1 h drug exposure
    cells grown drug-free for 3 days;
    2).   24 HA exposure followed by 1 h drug exposure
35 cells grown drug-free for 3 days; and
    3).   HA ± drug exposure for 1 hr, cells grown drug-
    free for 3 days.

Any detached cells will be tested for cell viability since it has been suggested that HA can play a pivotal role in cancer cell detachment and migration. If detached cells are viable the HA receptor status will be determined using FACS surface epitope identification. Similar experiments will be performed with short HA oligiosaccharides, ie: 4 sacc, 6 sacc, 12 sacc, 5600Da, 50,000Da, 100,000Da, 250,000Da.

These experiments will demonstrate the optimal HA:drug ratio *in vitro*, optimal HA exposure time and regimen, effect of HA molecular weight on adriamycin cytotoxicity.

After determining the optimal HA concentration, the IC_{50} of adriamycin will be used in a series of time course experiments to observe any effect of HA on adriamycin metabolism.

The [^{14}C] adriamycin will be exposed to the cells for 30min, 1h, 2h, 4h, 8h, 16h and 24h. The experimental conditions will be:

1). Exposure of cells to HA for 30 min followed by drug; and

2). Exposure of cells to HA for 24 followed by drug Co-exposure of HA/adriamycin.

Cells will be removed, hypotonically lysed and centrifuged at 113,000gav for 1 hr. The membrane pellet and supernatant will be counted and analysed for metabolites using HPLC.

Cells will also be grown on coverslips, where they will be exposed to adriamycin ± HA (exposures regimen as above) and a confocal photography time course will be used to track the cytosolic uptake and movement of the drug.

Identification of HA Receptors on MDR-1 positive and negative breast cancer cell lines, FACS quantitation of the CD44s, CD44v6, CD44v10 and RHAMM receptors will be conducted. Quantitation of the HA/receptor binding and saturation kinetics using FITC/HA and FACS analysis will

also be done.

By exposing the cells to:

1).   HA for 30 min followed by drug;

2).   HA for 24 h followed by drug; and

5 3).   HA/adriamycin

We will be able to determine any of these block CD44s and RHAMM receptors. The receptor status of any viable cells will be quantitated using surface epitope FACS analysis. If blocking of the HA receptors decreases the normally

10 observed synergism between adriamycin and HA, the membrane bound and cytosolic adriamycin will be quantited ± HA receptor blocking.

HA degradation by cell lines using [^{3}H]HA and gel filtration chromatography ± receptor blocking will be

15 studied.

HA of molecular weight, 4 sacc, 6 sacc, 12 sacc, 5600Da, 50,000Da, 100,000Da, 250,000Da, 750,000Da and 1,500,000 Da will be incubated with breast cancer cell lines at pre-determined "observed-effect" concentrations

20 and the following will be parameters investigated: Extracellular and intracellular calcium flux (cellular probe assays). Regulation of cytoskeletal components (micro-array of cytoskeletal genes), effect on volume of cells (Coulter size Analysis) and mobility of cancer cells

25 (Boyden Chamber matrigel assays) will also be conducted.

The effect of HA on the cell cycle will be undertaken by incubating HA of molecular weight, 4 sacc, 6 sacc, 12 sacc, 5600Da, 50,000Da, 100,000Da, 250,000Da, 750,000Da and 1,500,000 Da with breast cancer cell lines at

30 pre-determined "observed-effect" concentrations. Cells will be labelled with potassium iodide and subjected to FACS analysis. The number of cells in each stage of the cell cycle will be determined.

Comparisons of the *in vitro* efficacy of the

35 liposomal Doxorubicin and HA/Doxorubicin preparations will be conducted using the optimal HA/Doxorubicin preparation and the dosage range used by the Liposome Company in the

pre-clinical testing of the liposomal doxorubicin.

Section 2:

Before progression of the HA/adriamycin anti-

5   cancer therapy into Phase I human breast cancer trials it
is necessary to conduct preliminary toxicity experiments.
The experiments will focus on:
1).   Effect of hyaluronan on adriamycin uptake in
mouse body organs and fluids;

10   2).   Establish a preliminary dose range for adriamycin
Determine if HA targets adriamycin to human breast tumour
xenografts in nude mice;
3).   Compare the commercial liposomal Doxorubicin to
HA/doxorubicin uptake in mice; and

15   4).   Comparison of short-term efficacy of liposomal
doxorubicin and HA/doxorubicin.
From Inaba et al, (1988) the dose of adriamycin
in nude mice was 4 mg/kg which is a human equivalent dose
of $60mg/m^2$. Nude mice bearing human tumours will be

20   injected with adriamycin ± HA. Using adraimycin
concentrations of 4mg/kg ± 12.5mg/kg HA. The experimental
protocol will include the following treatment groups:
1).   4 mg/kg adriamycin;
2).   4 mg/kg adriamycin + 12.5mg/kg HA; and

25   3).   4 mg/kg liposomal doxorubicin.
Using adriamycin ± HA will be quantitatively
injected into the tail vein of the mouse.
At the time intervals of 2, 15, 30, 60min and
1.5, 2, 4, 8, 24 and 48h (4 animals/time point) the mice

30   will be killed by a 0.1ml IP injection of Nembutal.
All body organs, skeletal muscle, lymph nodes, bone marrow,
urine and blood will be removed and the adriamycin content
determined using HPLC and fluorescence.
Human breast tumours will be generated in nude

35   mice (WEHI CBA strain). The mice will be injected with:
1).   Mouse LD_{50} is 10mg/kg;
2).   4mg/kg adriamycin;

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3).   4mg/kg adriamycin + 12.5mg/kg HA;
4).   8mg/kg adriamycin;
5).   8mg/kg adriamycin + 12.5mg/kg HA;
6).   4 mg/kg liposomal doxorubicin;
5   7).   Saline; and
8).   12.5mg/kg HA.

The above mentioned will be quantitatively injected into the tail vein of the mouse (8 animals/group) on Days 2,4,6 of a weekly cycle.

10   Tumour volume, body mass, food intake and functionality of the mice will be monitored on a daily basis.

At the completion of the 8-week study the mice will be killed by a 0.1ml IP injection of Nembutal. All
15 body organs, tumour, skeletal muscle, lymph nodes, bone marrow, urine and blood will be removed processed for pathological assessment.

Section 3:

20   To answer some basic questions about the effect of HA anti-cancer therapy on colon cancer cells the following experiments should be conducted.

Investigation of the effect of HA/5-FU exposure times and concentration on drug-resistant and drug-
25 sensitive colon cancer cells.

Three resistant and 3 sensitive cell lines will be exposed to 5-FU at 1, 2.5, 5, 10, 20, 40, 60, 80 and 100nM, the following variables will be tested:
1).   1h drug ± 100nM HA exposure followed by 3 days of
30 drug-free growth;
2).   Constant drug exposure ± 100nM HA for 3 days;
3).   30 min 100nM HA exposure, followed by drug for 1h, cells grown drug-free for 3 days; and
4).   24h 100nM HA exposure, followed by drug for 1h,
35 cells grown drug-free for 3 days.

Using the IC_{50} of 5-FU as determined as above, HA (700kD) concentration will be varied to 1, 3, 10, 30, 100,

300nM, 1µM, 3µM, 10µM, 30µM and 100µM. The incubation
variables to be tested:

1).   30min HA exposure followed by 1 h drug exposure
cells grown drug-free for 3 days;

5   2).   24 HA exposure followed by 1 h drug exposure
cells grown drug-free for 3 days; and

3).   HA ± drug exposure for 1 hr, cells grown drug-
free for 3 days.

Any detached cells will be tested for cell

10   viability since it has been suggested that HA can play a
pivotal role in cancer cell detachment and migration. If
detached cells are viable the HA receptor status will be
determined using FACS surface epitope identification.

Similar experiments will be performed with short

15   HA oligiosaccharides, ie: 4 sacc, 6 sacc, 12 sacc, 5600Da,
50,000Da, 100,000Da, 250,000Da.

After determining the optimal HA concentration,
the IC_{50} of 5-FU will be used in a series of time course
experiments to observe any effect of HA on adriamycin

20   metabolism.

The [^{3}H] 5-FU will be exposed to the cells for
30min, 1h, 2h, 4h, 8h, 16h and 24h. The experimental
conditions will be:

1).   Exposure of cells to HA for 30 min followed by

25   drug; and

20.   Exposure of cells to HA for 24 followed by drug
Co-exposure of HA/5-FU.

Cells will be removed, hypotonically lysed and
centrifuged at 113,000gav for 1 hr. The membrane pellet

30   and supernatant will be counted and analysed for
metabolites using HPLC.

Cells will also be grown on coverslips, where
they will be exposed to 5-FU ± HA (exposures regimen as
above) and a confocal photography time course will be used

35   to track the cytosolic uptake and movement of the drug.

Identification of HA Receptors on resistant and
sensitive colon cancer cell lines, FACS quantitation of the

CD44s, CD44v6, CD44v10 and RHAMM receptors, Quantitation of HA/receptor binding and saturation kinetics using FITC/HA and FACS analysis will be done.

Blocking of CD44s and RHAMM receptors with
5 inhibitory antibodies, apply 5-FU $\pm$ HA following the protocols of:

1). Exposure of cells to HA for 30 min followed by drug; and

2). Exposure of cells to HA for 24 followed by drug
10 co-exposure of HA/5-FU.

Cells will be counted. The receptor status of any viable cells will be quantitated using surface epitope FACS analysis.

If blocking of the HA receptors decreases the
15 normally observed synergism between 5-FU and HA, the membrane bound and cytosolic 5-FU will be quantited $\pm$ HA receptor blocking.

HA degradation by cell lines using [^{3}H]HA and gel filtration chromatography $\pm$ receptor blocking will be
20 studied.

Effect of HA on the plasma membrane
Hyaluronan of molecular weight, 4 sacc, 6 sacc, 12 sacc, 5600Da, 50,000Da, 100,000Da, 250,000Da, 750,000Da and 1,500,000 Da will be incubated with breast cancer cell
25 lines at pre-determined "observed-effect" concentrations and the following will be parameters investigated:

1). Extracellular and intracellular calcium flux (cellular probe assays);

2). Regulation of cytoskeletal components (micro-
30 array of cytoskeletal genes);

3). Effect on volume of cells (Coulter size Analysis);

4). Mobility of cancer cells (Boyden Chamber matrigel assays);

35 5). Quantitation of HA receptors (FACS); and

6). Membrane potential (method to be determined).

An investigation of the role of HA neo-adjuvant

therapy on the inhibition of organ metastasis will be undertaken. In comparison to other treatment groups, mice receiving the HA therapy have demonstrated that:

1).   Reduced lymph node metastasis as compared to

5   other treatment groups;

2).   Inhibition of new tumour formation;

3).   Increased weight gain; and

4).   Enhanced well-being.

These results highlight the possible role of HA

10   anti-cancer therapy as an efficient means of reducing the spread of cancer. Through the obligatory choice of a pre-clinical model there is a restriction, whereby the spread of the secondary cancer normally occurs in the surrounding lymph nodes. It would be advantageous to use a model where

15   we can examine the spread of the cancer to every organ and the bone. By using a model known as the BAG vector metastasis model we would be able to monitor the spread of cancer to every organ and the bone.

In brief, the BAG vector consists of a neomycin-

20   resistant LacZ gene that can be stably transfected into human breast cancer cells. After intracardiac injections into the nude mice, followed by a 6-week treatment program it is possible to PCR detect the LacZ gene in any metastasizing cells/organs. Faxitron scanning with

25   detection of bone lesions would detect any bone metastasis.

The below treatments will be administered on Day1, Day 2 of a weekly cycle, for 6 weeks. The treatment groups (5 animals per group) will consist of:

1.   Saline

30   2.   30mg/kg 5-FU Day 1, Day 2;

3.   12.5mg/kg HA Day 1, Day 2;

4.   30mg/kg 5-FU + 12.5mg/kg HA (co-administered on Day 1, Day 2);

5.   12.5mg/kg HA on Day 1, 30mg/kg 5-FU on Day 2,

35   12.5mg/kg HA on Day 3, 30mg/kg 5-FU on Day 4;

6.   12.5mg/kg HA on Day 1,3;

7.   30mg/kg 5-FU on Day 2, 4;

8.   15mg/kg MTX Day 1, Day 2;
9.   15mg/kg MTX + 12.5mg/kg HA (co-administered on
Day 1, Day 2);
10.   12.5mg/kg HA on Day 1, 15mg/kg MTX on Day 2,
12.5mg/kg HA on Day 3, 15mg/kg MTX on Day 4;
11.   15mg/kg MTX on Day2, Day 4;
12.   15mg/kg MTX, 30mg/kg 5-FU, 30mg/kg cyclophosamide
on Day 1, Day 2; and
13.   15mg/kg MTX, 30mg/kg 5-FU, 30mg/kg cyclophosamide
+ 12.5mg/kg HA on Day 1, Day 2;
12.5mg/kg HA on Day 1, (15mg/kg MTX, 30mg/kg 5-FU, 30mg/kg
cyclophosamide) on Day 2, 12.5mg/kg HA on Day 3, (15mg/kg
MTX, 30mg/kg 5-FU, 30mg/kg cyclophosamide) on Day 4.

Neo-adjuvant Therapy:
          Immediately before intracardiac injection
administer the following:

1).   12.5mg/kg HA;
2).   15mg/kg MTX;
3).   15mg/kg MTX, 12.5mg/kg HA;
4).   30mg/kg 5-FU;
5).   30mg/kg 5-FU, 12.5mg/kg HA;
6).   15mg/kg MTX, 30mg/kg 5-FU, 30mg/kg
cyclophosamide; and
7).   15mg/kg MTX, 30mg/kg 5-FU, 30mg/kg cyclophosamide
+ 12.5mg/kg HA.
          Mouse mass and well being will be monitored daily
for 6 weeks. On completion of the treatment cycle, each
mouse will be scanned for bone lesions. After scanning
each organ and body fluid will be removed. A sufficient
cross section of the organ will be kept for possible future
pathological analysis, while the remaining tissue will be
homogenized and subjected to competitive PCR for the
detection of the LacZ gene.
          Any organs which exhibit metastasis will be
histologically processed and the pattern of colonization of

the cancer cells will be noted using galactosidase staining of the Lac Z gene.

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